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Determination of LSD and *N*-demethyl-LSD in urine by liquid chromatography coupled to electrospray ionization mass spectrometry

Harald Hoja[a], Pierre Marquet[a,*], Bernard Verneuil[a], Hayat Lotfi[a,b], Jean-Louis Dupuy[a], Gérard Lachâtre[a,b]

[a]*Department of Pharmacology and Toxicology, University Hospital, 87042 Limoges Cedex, France*
[b]*Laboratory of Toxicology, Faculty of Pharmacy, 87025 Limoges Cedex, France*

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Abstract

A sensitive and highly specific method for the determination of LSD and *N*-demethyl-LSD in urine, using combined liquid chromatography and mass spectrometry (LC–MS) with electrospray ionization, has been developed. Extrelut-3 extraction cartridges were used for a basic sample clean-up. Elution was obtained by toluene–diethyl ether (60:40, v/v). A Nucleosil C_{18} (150×1 mm I.D.) reversed-phase column was used for the chromatographic separation, together with a mixture of 2 m*M* ammonium formate buffer (pH 3) and acetonitrile (70:30, v/v) as mobile phase. Recoveries were 93 and 80%, detection limits 0.025 and 0.035 ng/ml for LSD and *N*-demethyl-LSD, respectively. Intra-assay precision, studied at four concentrations, was better than 9% at the ng/ml range and better than 14% at 0.10 ng/ml for both compounds. Limits of quantitation were 0.05 and 0.10 ng/ml for LSD and *N*-demethyl-LSD, respectively. Reproducibility was good and linearity excellent for LSD in the range from 0.05 to 20 ng/ml ($r>0.9999$, $n=7$).

Keywords: LSD; *N*-Demethyl-LSD; Lysergic acid diethylamide

1. Introduction

Lysergic acid diethylamide (LSD) is a highly potent psychedelic drug with a remarkably low toxicity. Of the four possible isomers and diastereomers of LSD, only D-LSD is pharmalogically active [1,2]. Iso-LSD, derived from LSD by epimerization on C_8, was sometimes found in LSD preparations [3] and has been shown to be also a sample work-up artifact formed in alkaline extraction media [4]. LSD is extensively metabolized in man (plasma half-life: $t_{1/2}=3.6$ h) to give *N*-demethyl-LSD (Nor-LSD, $t_{1/2}=10$ h), and 13- and 14-hydroxy-metabolites [5,6]. Only 1% of the drug, approximately, is excreted unchanged in urine [1,6]. Typical doses range between 20 and 80 μg, yielding plasma and urine concentrations at the sub-nanogram/milliliter level within a few hours after ingestion [7].

Identification and quantitation of LSD in body fluids has always been a challenging analytical problem for forensic or clinical toxicology laboratories. Reviews on the subject have been published

*Corresponding author. Address for correspondence: Service de Pharmacologie et Toxicologie, CHRU Dupuytren, 2 Avenue du Pasteur Martin-Luther-King, 87042 Limoges Cedex, France.

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[6–8]. The methods of choice for a rapid screening urine are RIA [9–11] and a recently introduced Syva EMIT [12] technique. Because of the more or less important cross-reactivities of these immuno-assays with LSD related compounds, metabolites or other drugs, positive results should be confirmed using separative techniques. This should be of particular concern for users of the EMIT technique for which false positive results in the presence of some neuroleptics and of other drugs have been reported [13]. Gas chromatography coupled to single mass spectrometry (GC–MS) or tandem mass spectrometry (GC–MS-MS) are most often used for determination or confirmation of LSD, in spite of the thermolability of the drug and of the need for derivatization [3,5,14–16]. Liquid chromatographic methods reported were HPTLC [9] and HPLC with fluorescence detection [4,11,17,18] but, although detection limits below 1 ng/ml could be attained, they lack specificity as compared to MS detection. Recently, workers used immunoaffinity chromatography coupled to electrospray mass spectrometry (ES–MS) [19], liquid chromatography–electrospray mass spectrometry (LC–ES-MS) [20,21] or tandem mass spectrometry (LC–ES-MS-MS) [22] and demonstrated the suitability of these approaches for the sensitive and specific detection of LSD in urine, as well as for metabolic studies.

On the basis of these preliminary results, a validated method for the quantitative determination of LSD and *N*-Demethyl-LSD in urine using LC–ES-MS with selected-ion monitoring (SIM) was developed.

2. Experimental

2.1. Chemicals and reagents

Lysergic acid diethylamide (LSD), lysergic acid diethylamide-d_3 (internal standard, I.S.) as well as a 8:2-mixture of *N*-demethyl-LSD and iso-*N*-demethyl-LSD were obtained as 0.1 g/l methanolic solutions from Radian (Austin, TX, USA) and stored at 4°C in the dark; HPLC-grade acetonitrile was obtained from Carlo Erba (Milan, Italy); sodium sulfate, diethyl ether and ammonium chloride were purchased from Prolabo (Paris, France); 25% ammonia solution and toluene of Uvasol quality were obtained from Merck (Darmstadt, Germany); high-purity formic acid and ammonium formate were purchased from Sigma (St. Louis, MO, USA). All reagents were of the highest available purity and used as received without further purification. Deionized water was prepared on a Milli-Q laboratory plant (Millipore, Bedford, MA). For sample extraction, Extrelut-3 cartridges (Merck) were used.

2.2. Standards and solutions

Stock solutions of LSD and *N*-demethyl-LSD were prepared at concentrations of 1 mg/l by dilution of the commercial solutions in methanol and stored at 4°C in the dark for a maximum of four weeks. The I.S. was prepared as a 0.1 mg/l methanolic solution. Working solutions for standard curves at concentrations of 2, 20 and 400 ng/ml were freshly prepared each day of analysis, by dilution in deionized water. Routine daily calibration curves were obtained by analyzing drug free urine samples fortified with 0.05, 0.1, 0.5, 1, 5, 10 and 20 ng/ml of each analyte. An extract of blank urine was also prepared for every calibration.

2.3. Sample preparation

To 2 ml of urine in a 15 ml glass tube were added 100 μl of I.S. and 1 ml of a saturated solution of ammonium chloride adjusted to pH 9.5 with ammonia. The tubes were briefly vortex-mixed (10 s) and the sample was applied to Extrelut-3 extraction cartridges. After 10 min of impregnation, elution of the analytes was realized with 12 ml of diethyl ether–toluene (6:4, v/v) into a clean set of 15 ml glass tubes. After addition of a spatula tip of sodium sulfate, the tubes were vortex-mixed and the dried supernatant transferred to 10 ml glass tubes. The solvent was evaporated at 30°C under a gentle stream of nitrogen. The dry extracts were redissolved in 25 μl of the mobile phase, of which 1 μl was injected into the chromatographic system.

2.4. HPLC conditions

The HPLC system consisted of a dual-piston syringe pump (Brownlee Labs, Santa Clara, CA,

USA). Samples were injected on a Rheodyne model 7410 injection valve equipped with a 1 μl internal loop (Rheodyne, Cotati, CA, USA). Chromatographic separation was performed on a Nucleosil C_{18} (150×1 mm I.D.) reversed-phase column (LC-Packings, Touzart and Matignon, Courtabœuf, France); the mobile phase was a mixture of 2 m*M* ammonium formate (pH 3)–acetonitrile (70:30, v/v), delivered at a flow-rate of 40 μl/min. All chromatographic solvents were filtered (0.46 μm) prior to mixing and degassed with helium thereafter.

2.5. Mass spectrometry

An API-100 Perkin Elmer-Sciex (Sciex, Foster City, Canada) atmospheric pressure ionization mass spectrometer was used, equipped with an electrospray-type Ionspray ionization device. Ultra-high purity nitrogen was used as nebulization and curtain gas. Calibration of the mass analyzer was performed by infusion (5 μl/min) of a commercial mixture of PPGs (polypropylene glycols, Applied Biosystems, Saint-Quentin-en-Yvelines, France) using a Harvard model 11 syringe pump (Harvard Scientific, South Natick, MA, USA) and monitoring eight *m/z* ratios in the 55 to 2300 a.m.u. mass range. Characteristic ions of LSD, *N*-demethyl-LSD and I.S. were identified, after chromatography of 1 mg/l pure solutions of the drugs, using full scan acquisition (*m/z* 65–400 u, step size 0.25 u) and successive orifice (OR) voltages of 20 and 70 V. Further optimization of the instrument parameters was done by direct infusion (40 μl/min) of the drugs diluted in the mobile phase. The main MS parameters were optimized for the protonated molecular ion of LSD (*m/z* 324.3, peak width=0.042 a.m.u.). Then the orifice voltage was optimized for each *m/z* ratio acquired in the SIM mode. The main parameter settings of the MS were as follows: nebulization gas flow 0.95 l/min; curtain gas flow 1.16 l/min; ionspray voltage 5000 V; electron multiplier voltage 1900 V.

2.6. Validation procedure

Recoveries were determined in triplicates at concentrations of 1, 10 and 20 ng/ml by extraction of urine samples fortified with LSD and *N*-demethyl-LSD but not with LSD-*d3*. Samples were treated as previously described and the dry extracts were redissolved in 25 μl of mobile phase containing I.S. Recoveries for the analytes were calculated by comparison of the peak area ratios with those of unextracted standard solutions of LSD, *N*-demethyl-LSD and I.S. representing 100% recovery.

For the analytical validation, the guiding principles established during a conference on "method validation for the quantitation of drugs in biological media" were followed [23]. The recommendations are briefly summarized as follows: (i) intra-assay (within-day) precision [24] should be studied at least at three concentrations. (ii) Intermediate (day-to-day) precision [24] should be assessed at least by five determinations of five to eight concentrations (excluding blank values). (iii) An acceptable precision, either within-day or intermediate, is characterized by a C.V. of less than 15% at every concentration and an acceptable accuracy by a deviation of less than 15% from the nominal value; at the limit of quantification (LOQ) C.V. and deviation from the nominal concentration of up to 20% are, however, acceptable.

Thus, intra-assay precision was assessed at concentration levels of 0.1, 1, 10 and 20 ng/ml, by extraction and analysis, on the same day, of six drug-free urine samples fortified with LSD and *N*-demethyl-LSD for each level. For the determination of intermediate precision, drug-free urine samples spiked at 0, 0.05, 0.1, 0.5, 1, 5, 10 and 20 ng/ml were prepared in advance in 10 ml volumetric flasks. Aliquots (2 ml) of these spiked samples were stored at −18°C until analysis. A set of calibrating samples was analyzed each day for five days and a calibration graph of the drug-to-internal standard peak area ratios of the pseudo-molecular ions, versus theoretical drug concentration, was constructed using a $1/x$ weighted least-squares linear regression analysis.

3. Results and discussion

ES ionization is a soft ionization technique [25–28] which is most efficient with compounds already ionized in solution [26,29–31] (i.e., basic compounds in LC–MS, as the mobile phase generally is moderately acidic). Protonated molecule ions $[M+H]^+$ or adducts of the molecule with ammonium ions are easily obtained for moderately basic compounds

like LSD (pK_a=7.5). Because of the softness of the ionization process fragment ion abundances are generally low, even for relatively fragile molecules. In single mass-analyzer instruments, a further fragmentation of fragile molecules by collision of ions with the ambient bath gas can be induced, using a convenient acceleration of gas phase ions at the entrance of the mass spectrometer [32]. With the instrument used in this study, this collision energy can be controlled by means of the orifice voltage.

Fig. 1 shows background-substracted mass spectra of LSD, acquired by full scan acquisition with respective orifice voltages of 20 V and 70 V. Using an orifice voltage of 20 V, LSD showed its $[M+H]^+$ as base peak at *m/z* 324.3 and probable fragment ions at 281.3 and 223.3 (Fig. 1a). The background-substracted spectrum acquired with an OR of 70 V shows *m/z* 208.0 as base peak and ions at *m/z* 180.3, 192.3, 197.3, 223.3, 251.3, 281.3 and 324.3. All these plus three additional ions have been found by other workers using tandem mass spectrometry for metabolic studies of LSD [22]. Full scan acquisition using the same orifice voltages gave *m/z* 310.3 as base peak and *m/z* 209 and 237.3 as fragments of confirmation for *N*-demethyl-LSD and *m/z* 327.3 as $[M+H]^+$ for LSD-*d*3.

An additional feature of the instrument is the possibility to attribute to each *m/z* ratio a different orifice voltage which permits to maximize, in a same acquisition schedule, transmission efficiencies for molecular ions and for fragments. The ions monitored during SIM acquisition as well as the corresponding optimized OR voltages are listed in Table 1. It should be noted, that *m/z* 208.0 and 281.3 could not be used as confirmatory ions for LSD, because I.S., which was LSD triply deuterated on the *N*-methyl group, gave fragment ions of the same *m/z* ratios. Thus, only *m/z* 223.3 could be used for confirmation of LSD.

Table 1
Orifice voltages (ORV) applied to and relative abundances of molecular and fragment ions of LSD and *N*-demethyl-LSD during selected ion monitoring acquisition (quantification ions are underlined)

m/z (u)	ORV (V)	LSD relative intensity (%)	*N*-Demethyl-LSD relative intensity (%)
209.0	110	5	27
223.3	90	31	–
237.3	70	–	13
310.3	20	–	<u>100</u>
324.3	20	<u>100</u>	–

Because of the small quantities which are expected

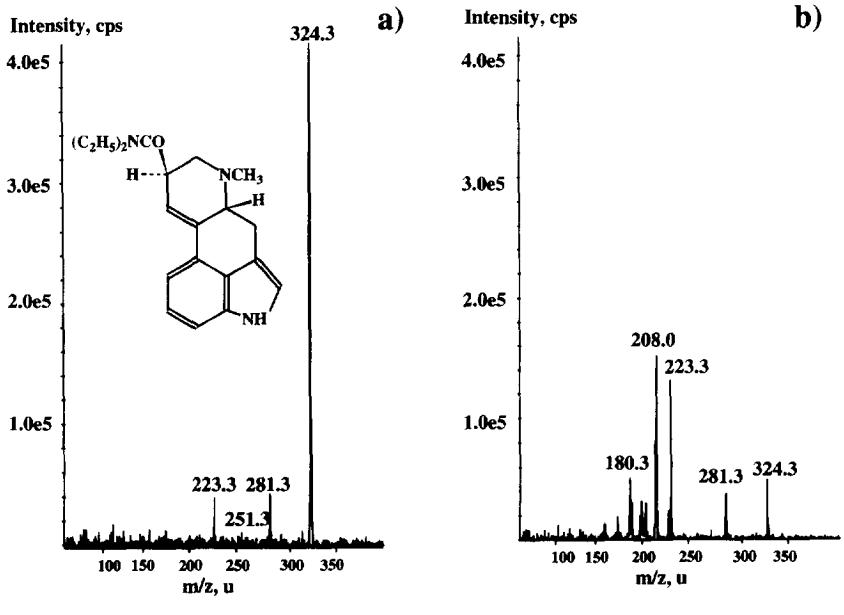

Fig. 1. Mass spectrum of LSD acquired with an orifice voltage of (a) 20 V and (b) 70 V.

to be found in urine samples, care was taken to maximize extraction recoveries and sample purities. Extrelut cartridges together with a selective elution solvent were expected to fulfill these requirements. Diethylether, methylenechloride and ethyl acetate have been unsuccessfully tried for the elution of the analytes from Extrelut cartridges: recoveries for LSD and *N*-Demethyl-LSD were satisfactory for all but a high chromatographic noise was also observed, due to coextracted endogeneous substances. Mixtures of these solvents with toluene were experimented in order to obtain purer extracts. A mixture of diethyl ether–toluene (6:4, v/v), yielding good recoveries and satisfying purity for both analytes, was finally chosen. Drying of the elution solvent with sodium sulfate prior to evaporation was found necessary because of the small quantities of water which are extracted in ether-based solvents. The recoveries determined at 1, 10 and 20 ng/ml were respectively 93.3 (C.V.=6.3%), 93.2 (C.V.=8.2%) and 98.2 (C.V.=4.1%) for LSD and 78.4 (C.V.=8.8%), 79.7 (C.V.=10.1%) and 84.4 (C.V.=4.3%) for *N*-demethyl-LSD.

Fig. 2 shows the selected ion currents of an extract of a positive urine sample, in which 4.3 ng/ml LSD and 0.3 ng/ml *N*-demethyl-LSD were determined. The respective retention times for LSD, *N*-demethyl-LSD and I.S. were 8.3, 7.8 and 8.3 min. Iso-LSD, not quantified in the assay, was also found (t_R 11.6 min). A neat baseline resolution for LSD and *N*-demethyl-LSD could not be achieved with the chromatographic system used. Nevertheless, proper identification and quantification of the analytes were possible because no interferences were observed.

Table 2 shows the intra-assay precision of the method. Precision of determination was excellent for LSD at all concentrations (C.V. lower than 9.0%, $n=6$). For *N*-demethyl-LSD, coefficients of variation were generally higher but satisfactory at all concentrations investigated.

The intermediate precision for LSD was excellent (see Table 3), with C.V. values lower than 14% and deviation from the nominal values of less than 6% at all concentrations. For *N*-demethyl-LSD, accuracy was satisfactory down to 0.05 ng/ml but at this concentration the C.V. was higher than 20%. Taking into account the previously outlined criteria, the lower LOQ were 0.05 ng/ml for LSD and 0.10 ng/ml for *N*-demethyl-LSD. Typical equations for

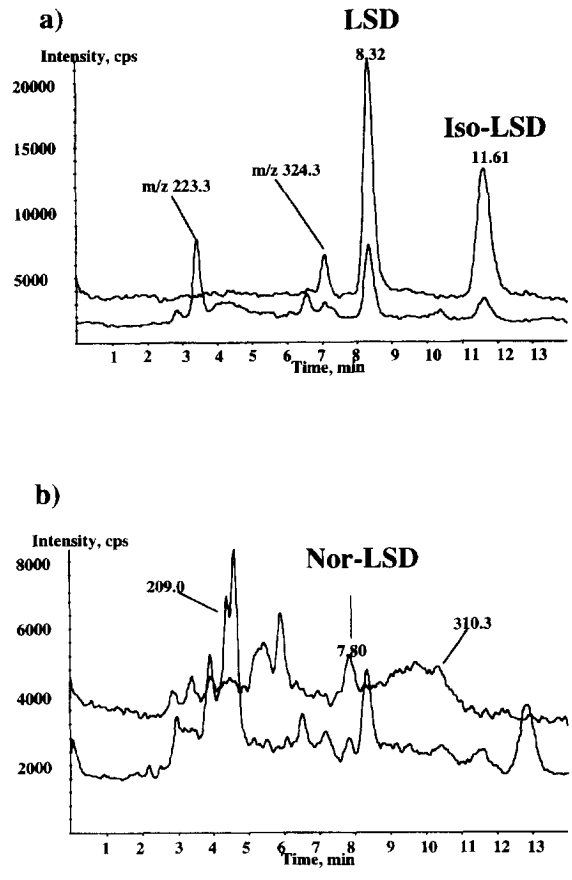

Fig. 2. Selected ion chromatograms of a positive urine sample. (a) LSD: *m/z* 324.3 (100%) and 223.3 (31%). (b) *N*-demethyl-LSD: *m/z* 310.3 (100%) and 209.0 (27%).

$1/x$ weighted regression analysis were $A(a)/A(I.S.)=0.1306x-0.0039$ for LSD and $A(a)/A(I.S.)=0.1245x-0.0041$ for *N*-demethyl-LSD where $A(a)/A(I.S.)$ is the peak area ratio of the analyte versus the internal standard and x the theoretical concentration. Correlation coefficients were higher than 0.999 for both compounds. Detection limits were determined as 24 pg/ml for LSD and 30 pg/ml for *N*-demethyl-LSD, using three times the standard deviation of the intercept ($n=5$). Because of the legal consequences arising from the presence of LSD in urine, the retention times of ions of quantitation are insufficient for a conclusive identification. In current forensic practice, relative abundances of confirmatory ions versus quantitation ions within ±20% to those of drug standards are also required [15]. Using these criteria, the limit for unambiguous identification was

Table 2
Intra-assay precision of LSD and *N*-demethyl-LSD determination in urine

Added concentration (ng/ml)	LSD ($n=6$)		*N*-Demethyl-LSD ($n=6$)	
	Mean found concentration (ng/ml)	Precision (C.V., %)	Mean found concentration (ng/ml)	Precision (C.V., %)
0.1	0.104	9.0	0.101	13.2
1	1.050	3.6	1.038	8.7
10	9.838	5.1	9.592	7.5
20	21.129	4.7	19.644	4.6

0.1 ng/ml for LSD and 0.25 ng/ml of *N*-demethyl-LSD. These results place the present method among the most sensitive methods using single mass-analyzers. LOQs were considerably lower than the 0.5 ng/ml obtained with GC–EI-MS [15] and comparable to those of GC–CI-MS [5,14]. Only GC–CI-MS-MS was more sensitive with LOQs at the low pg/ml level [3]. For LC–MS, encouraging preliminary results at the low nanogram/milliliter level were obtained by several workers using single quadrupole mass spectrometers [19–21], while a limit of detection of 50 pg/ml was reported for LSD, iso-LSD, and *N*-demethyl-LSD, using LC–ES-MS-MS [22].

The present method should be able to detect the drug in urine for at least 30–48 h post-dose. As a matter of fact, following administration of 1 μg/kg of LSD to human volunteers, urinary concentrations of 0.15 ng/ml were found at 22 h, using GC–CI-MS [5]. In a similar study, 0.1 ng/ml of LSD was found by RIA in urine after 48 h [15]. *N*-Demethyl-LSD, the plasma half-life of which is considerably longer than that of LSD, should be detectable for an even longer time. Additionally, hydroxy metabolites, excreted in urine in similar proportions [5,6,15], should increase the specificity of a LSD assay provided standards were commercially available for the assessment of their chromatographic and spectrometric behaviour.

4. Conclusions

The described method for quantitative determination of LSD and *N*-demethyl-LSD in urine is sensitive, specific, precise and reliable. Limits of quantitation are among the lowest published to date for single mass analyzing instruments. A limit of 0.1 ng/ml has been established for conclusive identification of LSD. The simple one-step extraction procedure permits to rapidly verify the presence of the drug or its metabolite in urine testing positive with either RIA or EMIT techniques, at usual cut-off limits of 0.5 ng/ml. Iso-LSD could also be identified by the presented technique but in the absence of a reference standard commercially available, its quantitation is not possible at the moment. An assay for serum and plasma determinations of LSD and *N*-demethyl-LSD is currently being studied. These

Table 3
Intermediate precision and accuracy of LSD and *N*-demethyl-LSD determination in urine

Added concentration (ng/ml)	LSD ($n=5$)			*N*-Demethyl-LSD ($n=5$)		
	Mean found concentration (ng/ml)	Precision (C.V., %)	Accuracy (%)	Mean found concentration (ng/ml)	Precision (C.V., %)	Accuracy (%)
0.05	0.047	11.3	94.7	0.059	24.9	118.8
0.10	0.100	4.9	99.7	0.107	14.8	106.7
0.50	0.502	12.5	100.5	0.472	12.1	94.4
1	1.074	7.6	107.4	0.925	9.1	92.5
5	4.813	4.9	96.3	4.295	8.1	85.9
10	10.176	3.1	101.8	9.549	5.2	95.5
20	19.938	1.7	99.7	21.242	3.4	106.2

encouraging results contribute to show that LC–ES-MS is a technique mature for routine applications in toxicological laboratories.

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